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African Swine Fever Virus EP153R Open Reading Frame Encodes a Glycoprotein Involved in the Hemadsorption of Infected Cells†

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The open reading frame EP153R, located within the *Eco*RI E′ fragment of the African swine fever (ASF) virus genome, is predicted to encode a membrane protein of 153 amino acids that presents significant homology to the N-terminal region of several CD44 molecules. EP153R contains multiple putative sites for N-glycosylation, phosphorylation, and myristoylation, a central transmembrane region, a C-type animal lectin-like domain, and a cell attachment sequence. Transcription of EP153R takes place at both early and late times during the virus infection. The disruption of the gene, achieved by insertion of the marker gene LacZ within EP153R, did not change either the *in vitro* virus growth rate or the virus-sensitive/resistant condition of up to 17 established cell lines, but abrogated the hemadsorption phenomenon induced in ASF virus-infected cells. As the sequence and expression of the ASF virus protein pEP402R, a CD2 homolog responsible for the adhesion of erythrocytes to susceptible cells, was unaffected in cultures infected with the EP153R deletion mutant, we conclude that the gene EP153R is needed to induce and/or maintain the interaction between the viral CD2 homolog and its corresponding cell receptor.

INTRODUCTION

African swine fever (ASF) virus, a large enveloped deoxyvirus, is the causative agent of an important disease of domestic pigs (reviewed in Hess, 1981; Viñuela, 1987; Costa, 1990). The virus contains a double-stranded DNA molecule ranging in size between 170 and 190 kbp depending on the virus strain (Blasco *et al.,* 1989) and about 34 structural proteins with molecular masses of 10 to 150 kDa (Carrascosa *et al.,* 1985). The ASF virus infects only species of the Suidae family and ticks of the *Ornithodoros* genus (Argasidae family) (Viñuela, 1985; Wilkinson, 1989). In domestic pigs the virus is associated mainly with tissue macrophages and blood monocytes (Malmquist and Hay, 1960; Plowright *et al.,* 1968) and to a lesser extent, with reticular cells, polymorphs, and megakaryocytes (Casal *et al.,* 1984; Wilkinson, 1989).

One characteristic feature of the cells infected with ASF virus field isolates is the ability to adsorb swine erythrocytes (hemadsorption) on its surface (Malmquist and Hay, 1960), a property successfully exploited to differentiate the ASF virus from other agents that produced diseases with symptoms likely to be confused with those observed in ASF (Hess and De Tray, 1960) and to develop specific techniques for ASF virus titration (Enjuanes *et al.,* 1976).

The analysis of the complete sequence of ASF virus strain BA71V (Yáñez *et al.,* 1995) has greatly expanded our knowledge about the possible role of many of the 151 open reading frames contained in the ASF virus genome. A virus protein, homologous to the CD2 adhesion receptor of T cells, encoded by the EP402R gene, has been directly involved in the hemadsorption phenomenon induced by the ASF virus in the infected cell (Rodríguez *et al.,* 1993). Another ASF virus-encoded protein that could be important in the control of the adhesion ability of infected cells is pEP153R, which contains a central transmembrane region, a C-type animal lectin-like domain, and a cell attachment (RGD) sequence (Yáñez *et al.,* 1995). In this report we present a comparison of the amino acid sequence of pEP153R and several CD44 molecules, along with experiments describing the transcriptional expression of the viral gene EP153R during infection of cultured cells. The disruption of the gene proved that protein pEP153R is required, in addition to the viral CD2 homolog pEP402R, to accomplish efficiently the process of hemadsorption in ASF virus-infected cells, revealing a role in the regulation (stabilization) of the interaction of CD2 with the corresponding cell receptor, as has been described for the CD2-mediated binding of human erythrocytes to human T cells (Haynes *et al.,* 1989).

† Dedicated to the memory of Eladio Viñuela.

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RESULTS

Properties of EP153R ORF

The EP153R ORF is located within the *Eco*RI E′ genomic fragment of the BA71V strain of the ASF virus (Fig. 1A) and encodes a protein, designated pEP153R, of 153 amino acids with a predicted molecular size of 18 kDa. The analysis of the sequence (Fig. 1B) revealed eight possible sites for N-glycosylation, three for phosphorylation, and two for myristoylation, in addition to the Arg-Gly-Asp (RGD) motif for integrin recognition. The hydropathy profile of pEP153R (Fig. 1C) resembled that of a typical class II transmembrane protein (Singer *et al.,* 1987) containing three domains: (i) a hydrophilic segment of 27 amino acids in the N-terminal end of the protein, (ii) a hydrophobic stretch (positions 28 to 50) that could act as a transmembrane domain, and (iii) a hydrophilic section of 103 residues in the C-terminal region of the polypeptide.

Transcriptional analysis of EP153R

Transcription of the EP153R ORF was studied by Northern blot and primer extension analyses. For these experiments, RNA was prepared from Vero cells either mock-infected or infected with ASF virus in the presence or in the absence of cycloheximide and citosine arabinoside, as described under Materials and Methods.

Northern blot hybridization was performed with a ^{32}P-end-labeled oligonucleotide complementary to the mRNA of EP153R. The probe recognized three RNA species of 0.7, 2.3, and 2.7 kb synthesized during the immediate-early and early phases of infection, and four transcripts of about 0.6, 1.0, 2.2, and 2.6 kb in the virus-induced late RNA sample (Fig. 2B).

To map the 5′-end of the EP153R transcription product, a primer extension analysis was performed, using as a primer the same oligonucleotide used in Northern blot hybridization. The results from the primer extension revealed a major product of 279 nucleotides in the samples of both immediate-early and early RNA, which corresponded to an initiation of transcription at position −164 relative to the first nucleotide of the EP153R ORF (Figs. 2A and 2C). A minor band of 179 nucleotides was detected with the late RNA sample, indicating the existence of a late mRNA initiating at 64 nucleotides upstream of EP153R (Figs. 2A and 2C). The transcripts of 0.7 and 0.6 kb, detected by Northern blot in the early and late RNA samples, respectively, would correspond to those detected in the primer extension if, as expected, their 3′-ends map at the first motif of 7 consecutive thymidilate residues (7T) (Figs. 2C and 2D), a sequence that has been found to be associated with the 3′-end formation of both early and late ASF virus mRNAs (Almazán *et al.,* 1992, 1993). In addition, the longer transcripts detected by Northern blot could be explained by the existence of read-through transcripts (from EP402R and EP153R) ending in different stretches of 7T (Fig. 2D).

Homology of protein pEP153R with C-type animal lectins and with the N-terminal region of CD44 molecules

A search of the data bases carried out with the FASTA program (Pearson and Lipman, 1988) revealed that protein pEP153R displayed significant similarity to proteins containing a C-type animal lectin-like domain (Yáñez *et al.,* 1995). The highest optimized FASTA scores were obtained with sequences on human (Hamann *et al.,* 1993) and mouse CD69 (Ziegler *et al.,* 1993), vaccinia virus proteins A40 (Amegadzie *et al.,* 1991) and A34 (Smith *et al.,* 1991), and rat and smallpox virus asyaloglycoprotein receptors (Fig. 3A). All the members of this family contain four highly conserved cysteine residues responsible for the formation of intramolecular disulfide bonds.

Moreover, a search of the protein data bases revealed a significant homology between pEP153R and the CD44 molecules involved in cellular adhesion and T-cell activation, as can be seen after a multiple alignment of the amino acid sequence of pEP153R and domains of the N-terminal region of members of the CD44 family from different origins (Fig. 3B).

Topology of protein pEP153R

To study the posttranslational processing of protein pEP153R, and its insertion and orientation into the membrane, the EP153R gene was cloned into the Bluescript plasmid under the T7 polymerase promoter. Protein pEP153R was then synthesized *in vitro* by a rabbit reticulocyte system either in the absence or in the presence of canine pancreatic microsomes, treated by proteinase K (±Triton X-100), and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). As shown in Fig. 4, in the absence of microsomes a major protein of about 18 kDa was synthesized, corresponding to the predicted size of the polypeptide encoded by EP153R. Translation in the presence of microsomes yielded a modified product of about 50 kDa, which should correspond to a glycosylated form of the protein, since eight possible sites for N-glycosylation were present in the C-terminal region of the sequence. Proteinase K treatment of the sample synthesized in the presence of microsomes rendered a product slightly less than 50 kDa in size, in contrast with the high level of sensitivity to protease exhibited by the protein produced in the absence of microsomes. After solubilization of microsomes with Triton X-100, the protease treatment resulted in the disappearance of bands ascribed to pEP153R. These observations indicate that a small segment of the N-terminal region of the protein is exposed to the action of proteases, while the C-terminal domain is

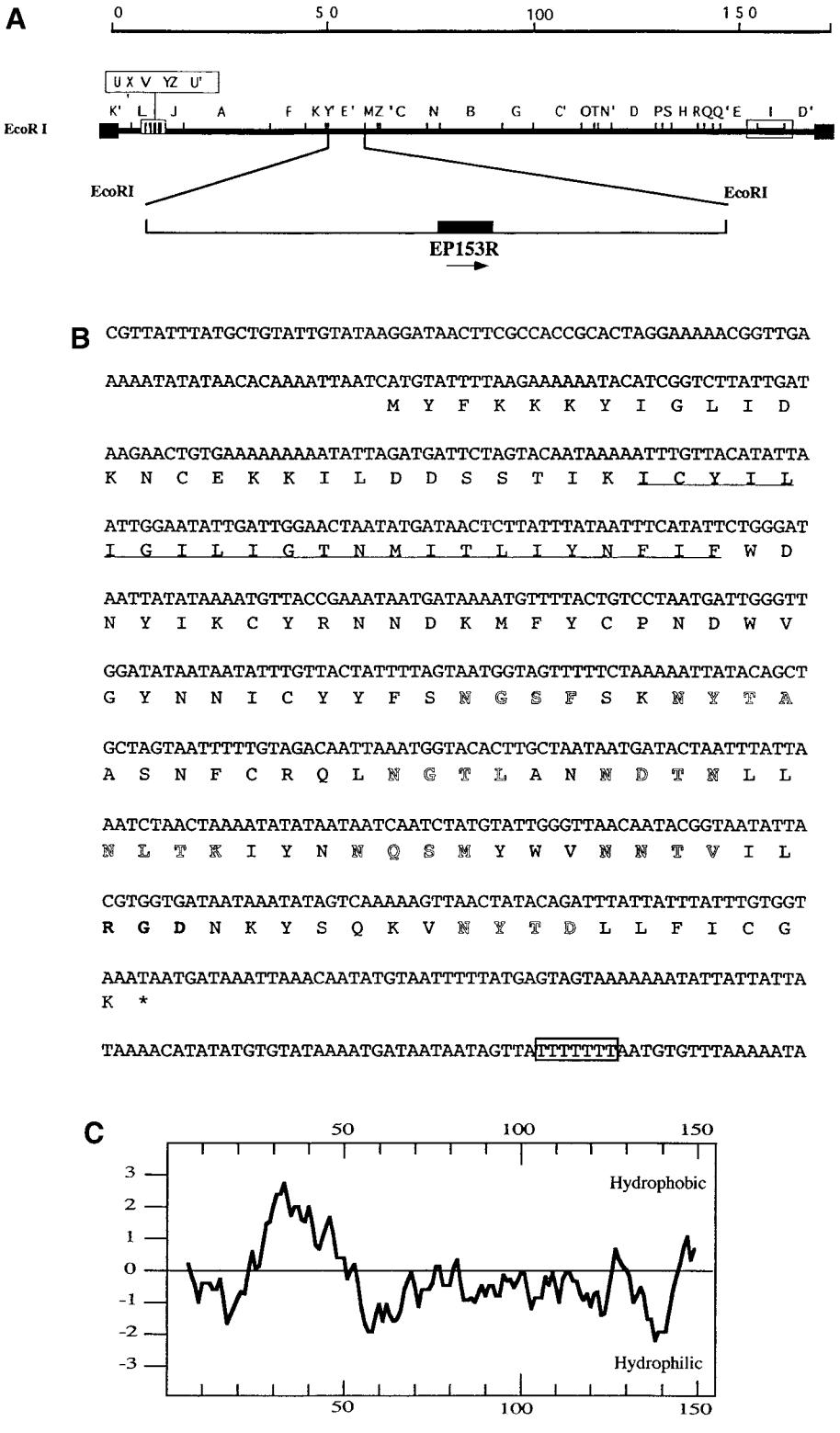

FIG. 1. Nucleotide and predicted amino acid sequence of EP153R ORF. (A) Localization in the ASF virus genome of restriction fragment *Eco*RI E′ containing EP153R ORF (indicated by an arrow). (B) Sequence of EP153R ORF. Residues corresponding to the putative transmembrane region are underlined. Potential sites for N-glycosylation are outlined and the RGD motif is highlighted in boldface type. The transcriptional termination motif (T_7) is boxed. (C) Graphic representation of the hydropathy profile of pEP153R obtained by the method of Kyte and Doolittle (1982).

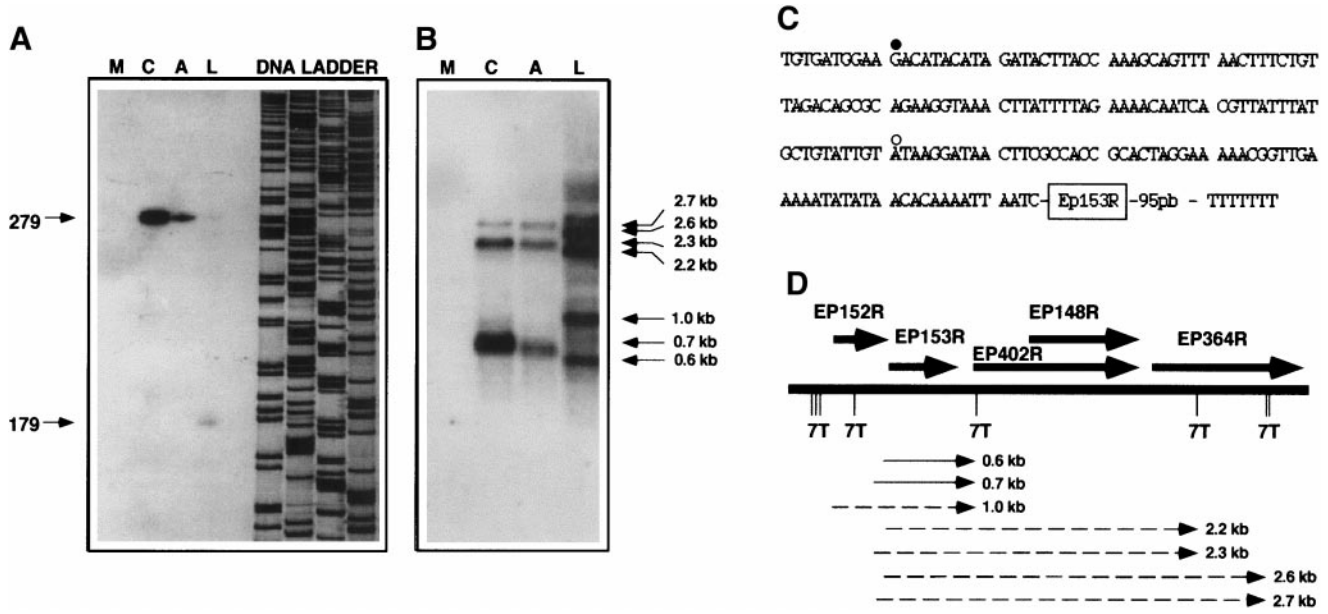

FIG. 2. Transcriptional analysis of EP153R. (A, B) Autoradiograms of primer extension (A) and RNA hybridization (B) of mock-infected cells (lanes M) and ASF virus-induced immediate-early (lanes C), early (lanes A), and late RNA (lanes L) are shown. Samples in A were separated alongside an irrelevant DNA sequencing reaction (DNA ladder) used as a size marker. The size of the relevant DNA fragments is indicated. (C) Precise location of early (●) and late (○) transcription initiation sites. (D) Map of EP153R mRNAs (thin arrows) within the *Eco*RI E′ fragment of the ASF virus genome. Transcripts of 1 to 2.7 kb are represented by dashed arrows. The positions of ORFs (heavy arrows) and the distribution of stretches of seven or more consecutive thymidilate residues (7T) are also indicated.

protected by the microsomes, supporting the assumption of pEP153R being a class II integral membrane glycoprotein with an extracellular C-terminus.

Construction of an ASF virus deletion mutant lacking EP153R gene

A common approach to study the function of a particular protein is the generation of virus mutants in which the gene encoding for that polypeptide is partially or totally deleted. To analyze the role of the EP153R gene product in the ASF virus life cycle, a virus recombinant lacking the EP153R gene, vΔEP153R, was generated by *in vivo* homologous recombination, using the deletion plasmid pΔEP153R described under Materials and Methods. This vector was designed to facilitate the replacement of a genomic DNA fragment of 333 bp, covering the majority of EP153R, with the marker gene LacZ under the control of the ASF virus promoter p72 (Figs. 5A and 5B). Deletion mutant viruses were selected and purified as previously described (Rodríguez *et al.,* 1992). To ensure that the vΔEP153R genome was as expected, DNA from the parental virus, BA71V, and DNA from the recombinant virus, vΔEP153R, were purified from infected Vero cells, digested with *Eco*RI restriction enzyme, and analyzed by DNA hybridization using two [α-^{32}P]ATP labeled probes: (i) the EP153R gene, obtained from the Bluescript-EP153R plasmid digested with *Bam*HI and *Eco*RI restriction enzymes, and (ii) the LacZ gene obtained from the pINSβgal plasmid (Rodríguez *et al.,* 1992) digested with *Bam*HI restriction enzyme. As expected, the EP153R probe recognized one *Eco*RI fragment of 8.9 kb in the sample corresponding to BA71V and two *Eco*RI fragments of 7.5 and 4.1 kb in the sample corresponding to vΔEP153R, while the LacZ probe recognized the two *Eco*RI fragments of 7.5 and 4.1 kb in the sample corresponding to vΔEP153R and nothing in the sample corresponding to the parental virus (Fig. 5C). These results demonstrated that the genomic structure of the ASF virus deletion mutant was as predicted.

Identification of the EP153R polypeptide in ASF virus-infected cells

To analyze the expression of pEP153R during the virus infection cycle, a rabbit antiserum was raised against a synthetic peptide specific for this protein (Materials and Methods). First we studied the kinetics of pEP153R expression in infected cells. Vero cell cultures were infected with BA71V and collected at different times after virus infection, to be analyzed by Western blot using the anti-pEP153R antiserum (Fig. 6A). As expected from the *in vitro* expression data, a protein of about 50 kDa was specifically recognized from 6 hpi and accumulated up to 22 hpi. The protein pEP153R was also detected at 16 hpi in the presence of citosine arabinoside, although in a lesser amount than in the absence of inhibitor, indicating that the protein was expressed at both early and late times in the infection virus cycle, in agreement with the results obtained by Northern blot and primer extension

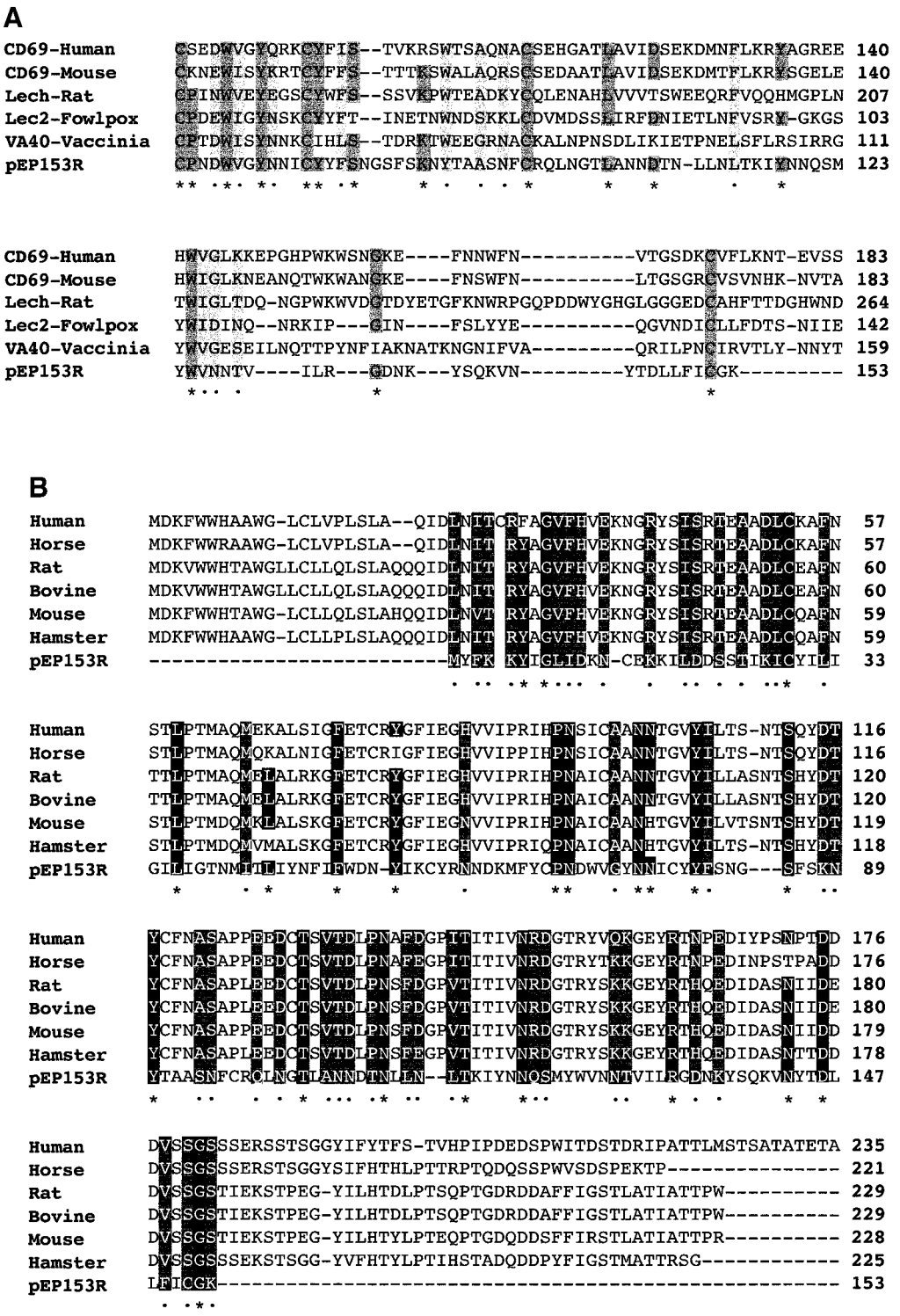

FIG. 3. Homology of protein pEP153R with C-type animal lectins and with CD44 molecules. (A) Multiple alignment of pEP153R (aa 67 to 153) with partial sequences of human and mouse CD69, vaccinia virus protein A40, and rat and smallpox virus asyaloglycoprotein receptors. (B) Multiple alignment of pEP153R with the N-terminal region of human, horse, rat, bovine, mouse, and hamster CD44 molecules. The alignments were generated by using the ClustalW program. The numbers on the right indicate the amino acid positions. Residues in EP153R identical to those in at least three molecules (asterisks) or equivalent in all of them (dots) are indicated.

experiments (Fig. 2). Considering the data obtained by *in vitro* expression, and the presence of eight putative sites for N-linked carbohydrates in pEP153R, the glycosylation of the protein was also investigated. Cultures of Vero cells were infected with either BA71V or vΔEP153R, in the presence or in the absence of tunicamycin, and collected

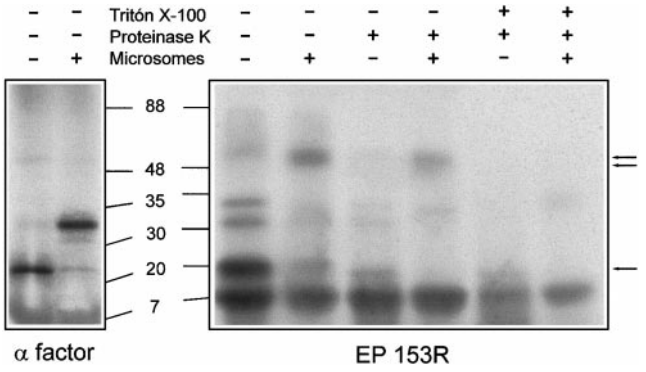

FIG. 4. *In vitro* transcription and translation of the EP153R gene. For analysis of cotranslational processing, canine pancreas microsomes were added to the reaction mixture. Samples were treated with proteinase K, in the absence or in the presence of Triton X-100, and analyzed by SDS–PAGE. The migration position of molecular size markers (in kDa) are indicated. Major gene products are indicated by arrows on the right. Control samples of glycosylation of α-mating factor gene from *S. cerevisae* (instead of viral EP153R gene) are shown on the left.

at 24 hpi to be analyzed by Western blot with the anti-pEP153R antiserum (Fig. 6B). As expected, the protein of ~50 kDa recognized in samples of BA71V-infected Vero cells was replaced, in the presence of tunicamycin, by a polypeptide with the predicted size (18 kDa) for the EP153R primary translation product, which might correspond to the nonglycosylated form of the protein. Accordingly, this protein was not found in cells infected with vΔEP153R, demonstrating that the gene had been efficiently inactivated. These analyses revealed that the EP153R gene encodes a glycoprotein present at both early and late times during virus infection.

EP153R is not required for *in vitro* replication of ASF virus

The achievement of a deletion mutant in EP153R gene is a fair proof of its nonessentiality for *in vitro* virus infection. In fact, the growth curves obtained either in Vero cells or in swine macrophages with both vΔEP153R and BA71V were equivalent when titrated by plaque assay (data not shown). To test the possibility that the EP153R gene could be involved in the determination of host range and tissue tropism, we infected up to 17 cell lines from different sources with either vΔEP153R or BA71V and compared the infective virus production at 24 hpi in each system. As shown in Fig. 7, only minor differences were observed in the total virus productivity estimated for both viruses in each cell, and all the lines maintained their characteristics of sensitivity or resistance to the ASF virus when infected with the EP153R deletion mutant.

Protein pEP153R is required to develop the hemadsorption process in ASF virus-infected cells

In preliminary experiments it was observed that the virus produced in swine macrophage cultures infected by vΔEP153R was very difficult to titrate by hemadsorption assay, although the virus cycle seemed to proceed correctly as estimated by the development of cytopathic

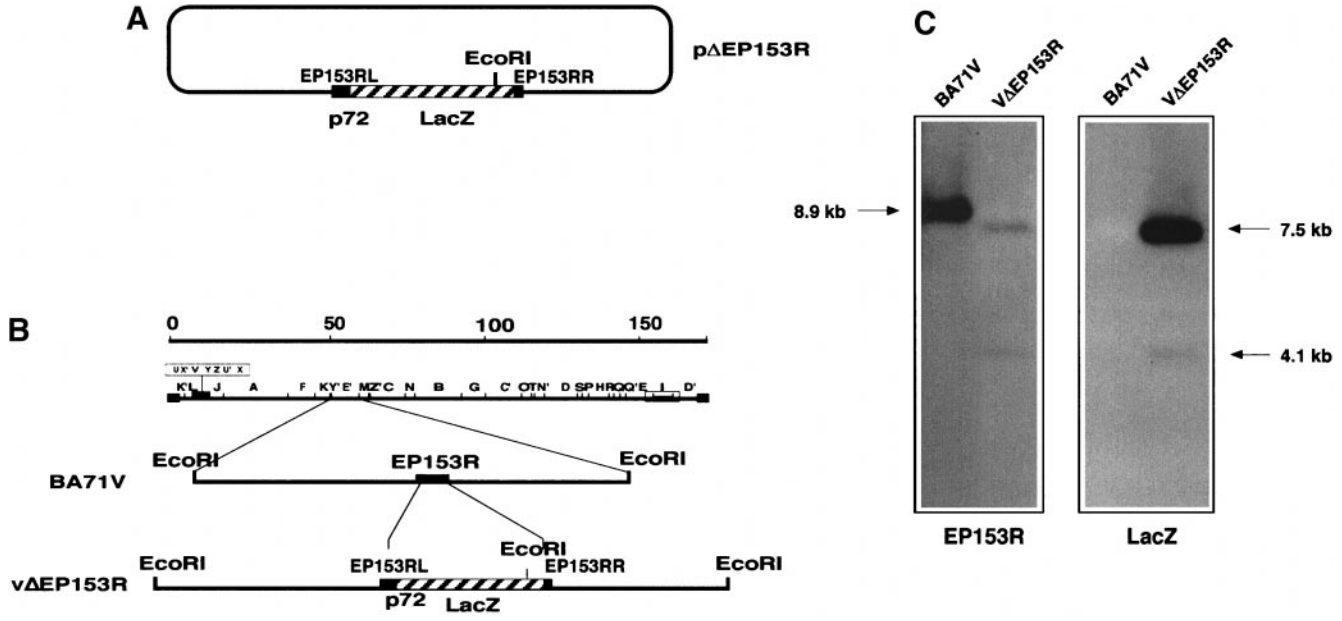

FIG. 5. Characterization of the genomic structure of the ASF virus recombinant vΔEP153R. (A) Deletion plasmid for the EP153R gene. (B) Genomic structure of the parental virus BA71V and that predicted for the recombinant vΔEP153R. The insertion point of the LacZ gene (striped box), fused to the virus promoter p72 (gray box), into the EP153R ORF (solid box), is indicated. (C) Genomic analysis of recombinant vΔEP153R. DNA purified from Vero cells infected with either BA71V or vΔEP153R was digested with *Eco*RI, subjected to agarose gel electrophoresis, and blotted onto nitrocellulose filters, before hybridization to ^{32}P-labeled probes corresponding to EP153R or LacZ genes. The sizes of detected DNA fragments are indicated.

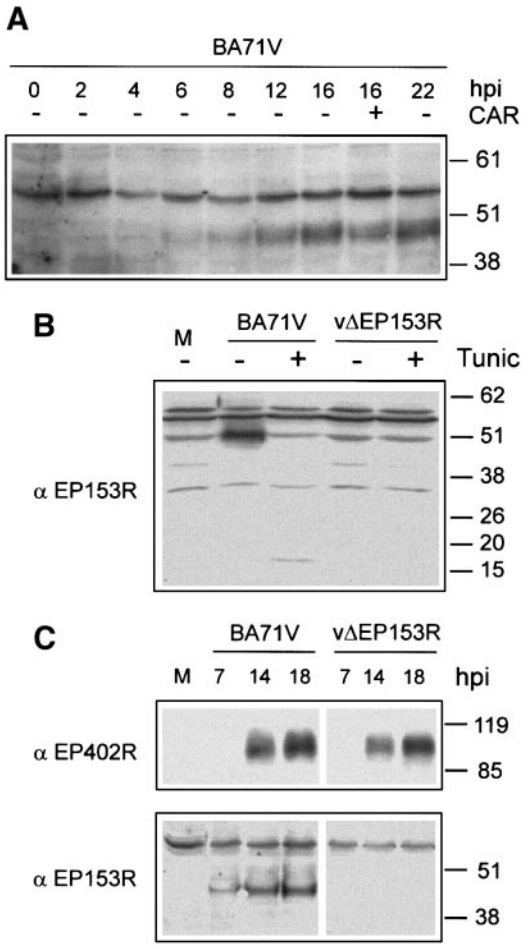

FIG. 6. Expression of pEP153R and pEP402R. (A) Kinetics of expression of pEP153R in infected cells. Vero cell cultures were infected with BA71V at a m.o.i. of 5 PFU per cell, in the presence or in the absence of citosine arbinoside (CAR), collected at different times after infection, and analyzed by Western blot with anti-pEP153R serum, as described under Materials and Methods. (B) Glycosylation of pEP153R in infected cells. Vero cells were mock-infected (M) or infected with either BA71V or vΔEP153R, in the presence (+) or in the absence (−) of 1 μg/ml tunicamycin (Tunic). Cell extracts were prepared at 24 hpi and analyzed by Western blot with anti-pEP153R serum. (C) Kinetics of expression of pEP402R and pEP153R in infected cells. Vero cells were mock-infected (M) or infected with either BA71V or vΔEP153R, collected at different times after infection, and analyzed by Western blot with either anti-pEP402R or anti-pEP153R serum. The migration positions of molecular size markers (in kDa) are indicated.

effect and the infective virus production evaluated by plaque assay. To study the capacity of vΔEP153R to induce the hemadsorption process, Vero cells and swine macrophages were infected with either vΔEP153R or BA71V. At 12 hpi swine erythrocytes were added to the cultures and the formation of hemadsorption rosettes on infected cells was then monitored by light microscopy until 3 to 4 days after infection. At 24 hpi nearly all the cells in cultures infected with BA71V were covered by a considerable number of erythrocytes (Figs. 8C and 8D), a process that was specific for ASF virus-infected cells (see the mock cultures in Figs. 8A and 8B). Infection of sensitive cells with vΔEP153R resulted in a drastic reduction both in the percentage of cells showing hemadsorption and in the number of erythrocytes per cell in the rosette (Figs. 8E and 8F), which almost disappeared when infection was allowed to proceed until 72 hpi. These observations were extended to a number of ASF virus-sensitive cell lines (CV2, LLCMK2, and BHK) with similar results (not shown).

It has been described previously that the EP402R gene of the ASF virus, which encodes a protein homolog of the adhesion molecule CD2, is responsible for the hemadsorption phenomenon in virus-infected cells (Rodríguez *et al.,* 1993). As the EP402R gene is contiguous with the EP153R gene in the ASF virus genome (Fig. 2D), we analyzed the nucleotide sequence of a region in the vΔEP153R genome including the EP402R ORF, 140 nucleotides upstream from the initiation codon and 200 nucleotides downstream from the termination codon. The results confirmed that the region sequenced was identical in both BA71V and vΔEP153R viruses. We also analyzed the expression of the EP402R gene in Vero cells infected with either BA71V or vΔEP153R, using a pEP402R-specific antiserum (F. Almazán, manuscript in preparation). As shown in Fig. 6C, the specific antiserum recognized a protein that was expressed with kinetics and at a level similar to those in cells infected with either the BA71V parental virus or the deletion mutant vΔEP153R. As a control, the same extracts were probed in parallel against the pEP153R antiserum, confirming the presence of pEP153R from 7 hpi in samples infected with BA71V and its absence in cultures infected by vΔEP153R. Minor differences in pEP402R band intensity could be explained by slight variations in the total protein loaded on the gel, as can be noticed by comparing the signal of a protein band of about 55 kDa that was recognized in all the samples by the pEP153R antiserum (Fig. 6C, bottom). Considering that the sequence of the EP402R gene (and nearby regions) was identical in both BA71V and vΔEP153R isolates, and that the protein pEP402R was correctly expressed during infection with recombinant virus vΔEP153R, we conclude that the deletion of the EP153R gene resulted in the loss of the ability of the ASF virus to induce the hemadsorption phenomenon.

DISCUSSION

The EP153R gene, located within the *Eco*RI E′ fragment of the BA71V strain of ASF virus, encodes a polypeptide of 153 amino acids, predicted to be a class II transmembrane protein. The presence of eight putative sites for N-glycosylation indicates that the mature protein is likely to be highly glycosylated and that its apparent size will probably be larger than predicted. In fact, *in vitro* synthesis of pEP153R in a rabbit reticulocyte system in the presence of canine microsomes generated a protein

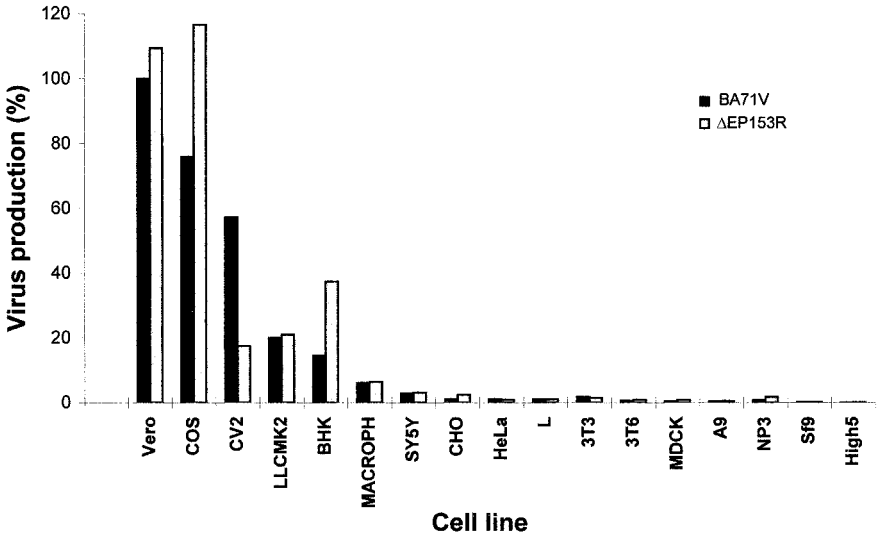

FIG. 7. ASF virus productivity on cell lines infected with BA71V or vΔEP153R. Duplicate cultures of different cell lines were infected with either BA71V or vΔEP153R, and infective virus production was titrated by plaque assay at 24 hpi. Data are represented as percentage of the virus produced in BA71V-infected Vero cell cultures.

of about 50 kDa, which turned to 18 kDa in the absence of posttranslational modifications. A similar behavior was observed in BA71V-infected Vero cell extracts produced in the absence or in the presence of tunicamycin and analyzed by Western blot with an antiserum specific for pEP153R: a polypeptide of 18 kDa, synthesized when glycosylation was inhibited, changed to 50 kDa when the infection was performed in the absence of tunicamycin. On the other hand, results obtained by proteinase K treatment indicated that only a portion in the N-terminal region of the protein was exposed to the protease action, while the C-terminal domain was protected by the microsomes, suggesting that pEP153R is a class II integral membrane glycoprotein with a C-terminal region exposed to the extracellular environment.

The EP153R gene is transcribed both during the early and the late phases of the infectious cycle, with two different transcription initiation sites, one for late transcription, close to the translation initiation codon, and the other for early transcription, at 164 nucleotides from the translation initiation codon. As expected from these results, by using an antiserum specific for a pEP153R peptide, we could detect the pEP153R at early times (6 hpi) in BA71V-infected Vero cells and its subsequent accumulation until 22 hpi.

The comparative analysis of sequences in the data bases showed that pEP153R contains a C-type animal lectin-like domain and a cell attachment (RGD) sequence, as previously described (Yáñez *et al.,* 1995). In addition, a significant homology was observed between pEP153R and the N-terminal region of CD44 molecules involved in cellular adhesion and T-cell activation. This hydrophilic N-terminus contains the hyaluronic acid binding domain (Peach *et al.,* 1993). Antibodies against CD44 have been shown to inhibit the interaction between human and sheep erythrocytes and T-lymphocytes (Hale *et al.,* 1989; Shimizu *et al.,* 1989; Sugimoto *et al.,* 1994), a phenomenon known as E-rosette formation, generated by the interaction between CD58 (LFA-3) and CD2 molecules present in the red and white cells, respectively. It has been suggested that the physical proximity of CD44 to erythrocyte LFA-3 molecules may be required to regulate the LFA-3–CD2 interaction (Haynes *et al.,* 1989). In the case of ASF virus-induced hemadsorption, a viral CD2 homolog, encoded by the EP402R gene, is responsible for the adhesion of swine erythrocytes to infected cells (Rodríguez *et al.,* 1993); however, the corresponding ligand in the red blood cell is unknown.

A viable deletion mutant lacking the majority of the EP153R ORF was constructed. No differences in growth kinetics or virus production were detected when the parental virus and the deletion mutant were compared, providing evidence of the nonessentiality of the EP153R gene for *in vitro* ASF virus infection. Dispensable genes, however, can be involved in the enhancement of virus infectivity, for example, by protecting the virus particles from the host response or by extending the host range and tissue tropism in *in vivo* infections. The results obtained when comparing total virus productivity in 17 established cell lines from various sources, infected by either BA71V or vΔEP153R, did not reveal any evidence of the possible involvement of the EP153R gene in the determination of cell susceptibility to the ASF virus. However, in the process of evaluation of virus yields by hemadsorption assays, a considerable difficulty was noted in the development of rosettes around cells infected by vΔEP153R. The results presented in this paper proved that the inactivation of the EP153R gene induced a drastic reduction both in the proportion of infected cells

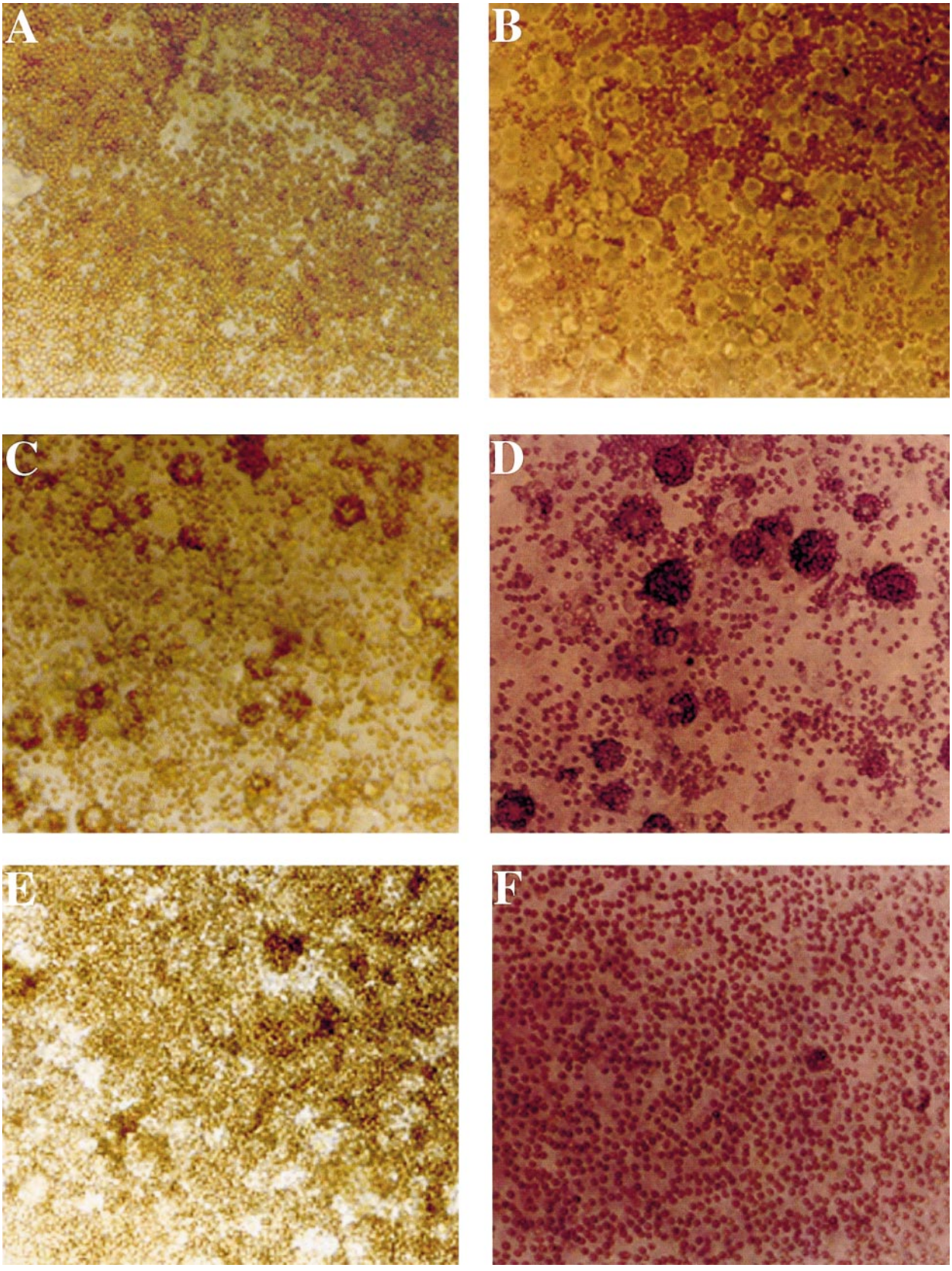

FIG. 8. Effect of EP153R gene deletion on the hemadsorption induced by ASF virus. Cultures of Vero cells (A, C and E) and swine alveolar macrophages (B, D and F), mock-infected (A and B) or infected with either BA71V (C and D) or vΔEP153R (E and F), were incubated with swine erythrocytes to develop the hemadsorption process on virus-infected cells.

surrounded by swine erythrocytes and in the number of erythrocytes attached per infected cell. It has been previously described that disruption of the EP402R gene, which encodes a CD2 homolog and is contiguous with the EP153R gene, also inhibits the hemadsorption phenomenon induced by ASF virus infection (Rodríguez *et al.,* 1993). Our results showed that the sequence of the EP402R gene in the deletion mutant vΔEP153R was identical to that in the parental virus BA71V and that the kinetics and level of expression of the viral CD2 homolog were equivalent in cells infected either with BA71V or with vΔEP153R, indicating that the CD2 homolog was properly synthesized by the recombinant virus. Previous results in our laboratory stated that the expression of the LacZ gene, inserted into the thymidine kinase gene of the ASF virus genome, had no inhibitory effects on the induction of hemadsorption (Rodríguez *et al.,* 1993). In addition, and in contrast to EP402R, expression of the EP153R gene as a recombinant product in heterologous eukaryotic systems, like Baculovirus or Sindbis pseudovirus, did not induce the hemadsorption process (data not shown).

Considering all the data presented here, our conclusion is that pEP153R is probably involved in the hemadsorption induced in ASF virus-infected cells, presumably as a stabilizer of the interaction between the viral CD2 homolog exposed in the virus-infected cell plasma membrane and its corresponding ligand in swine erythrocytes. This type of hemadsorption-negative virus mutant represents a useful and convenient tool with which to understand the biological relevance of the hemadsorption process in *in vivo* ASF virus infections, which manifest, among other characteristics, several hemostatic abnormalities and consumption coagulopathies associated with the disease (Villeda *et al.,* 1993a,b). Further characterization of proteins like pEP153R, which may interact with erythrocytes, lymphocytes, monocytes, adhesion molecules related to the immune system, platelets, cellular integrins, and caspases (via RGD sequence), will help us to understand the biology of the ASF virus infection.

MATERIALS AND METHODS

Cells and viruses

All the established cell lines used throughout this report were seeded from working stocks kept in storage in our laboratory and can be obtained from the American Type Culture Collection, with the exception of SY5Y (Biedler *et al.,* 1978) and BHK (Invitrogen). Swine alveolar macrophages were prepared by broncho-alveolar lavage as described (Carrascosa *et al.,* 1982). Mammalian cells were cultured at 37°C in Dulbecco's modified Eagle's (DME) medium supplemented with 5% newborn calf serum (for Vero cells), 10% homologous swine serum (for swine macrophages), or 5% heat-inactivated fetal calf serum (for the rest of the cells). Insect cells were maintained at 27°C in TC100 medium supplemented with 10% heat-inactivated fetal calf serum.

The Vero-adapted ASF virus strain BA71V was propagated and titrated either by plaque assay on Vero cells or by hemadsorption on alveolar swine macrophages, as previously described (Enjuanes *et al.,* 1976; Carrascosa *et al.,* 1982). The ASF virus deletion mutant vΔEP153R was constructed as described below.

Assay of virus production

To assay the ASF virus production in different established lines, cell cultures were infected in their corresponding medium with either BA71V or vΔEP153R at a m.o.i. of 10 PFU per cell. After virus adsorption inoculum was removed, cell cultures were washed twice with warm medium and then incubated at 37°C. Total virus was collected at different times after infection and titrated either by plaque assay on Vero cells or by hemadsorption on alveolar swine macrophages, as indicated (Enjuanes *et al.,* 1976).

Preparation and analysis of RNA

Whole-cell RNA was prepared by the TRI-Reagent (Molecular Research Center, Inc.) method (Chomczynski, 1993) from mock-infected cells from cells infected with ASF virus (5 PFU per cell) for 8 h in the presence of either 40 μg/ml of cycloheximide (immediate-early RNA) or 100 μg/ml of citosine arabinoside (early RNA), and from cells infected for 16 h in the absence of drugs (late RNA).

Both the Northern blot and the primer extension analyses were performed as previously described (Rodríguez *et al.,* 1994) using a ^{32}P-end-labeled oligonucleotide (5′-TTCCAATCAATATTCCAATTAATATGTAAC-3′), complementary to nucleotides 90 to 118 of the coding strand of EP153R.

For Northern blot, 15 μg of each of the different RNAs was fractionated on formaldehyde agarose gels, transferred to nitrocellulose, and hybridized to the ^{32}P-labeled probe. For primer extension, after hybridization of the 5′-end-labeled primer to 20 μg of the different RNAs, the samples were extended with avian myeloblastosis virus reverse transcriptase for 2 h at 37°C and then subjected to electrophoresis in 6% polyacrylamide sequencing gels.

Purification and analysis of DNA

Purification of ASF virus DNA was carried out as described (Enjuanes *et al.,* 1976). Plasmid DNA purification, endonuclease restriction analysis, DNA hybridizations, and preparation of radioactive probes were performed using standard protocols (Sambrook *et al.,* 1989). DNA sequencing was carried out after PCR amplification of selected regions of the viral genome by using dideoxinucleotide terminators (Sanger *et al.,* 1977).

Plasmid construction

The deletion vector pΔEP153R was constructed to facilitate the replacement of a genomic DNA fragment of 333 bp within the EP153R ORF with the *Escherichia coli* marker gene LacZ fused to the ASF virus promoter p72. A 820-bp *Hin*fI fragment, containing the 5′ end of the EP153R ORF, was obtained from plasmid pBA71V-CD2 (Rodríguez *et al.,* 1993), treated with Klenow enzyme, and cloned into *Sma*I-cut p72GAL10T (García *et al.,* 1995) to generate plasmid p72GAL10TL. A 1377-bp *Bsa*AI fragment, containing the 3′ end of EP153R, was also obtained from plasmid pBA71V-CD2 and cloned into p72GAL10TL, which had been previously cut with *Sal*I, and treated with Klenow enzyme to generate the deletion vector pΔEP153R (Fig. 5A).

In vitro transcription and translation

Expression of the EP153R gene *in vitro* was done by coupled transcription and translation in the TNT reticulocyte lysate system (Promega). The reaction was carried out using [^{35}S]Met/Cys Translabel Mix (Amersham) at 30°C for 90 min, in the presence or in the absence of canine pancreatic microsomes (Promega) to assess posttranslational processing of the EP153R gene product. Positive controls of glycosylation with the α-mating factor (instead of EP153R) gene from *Saccharomyces cerevisae* were run in parallel. Posttranslational proteolysis was performed using 0.5% Proteinase K for 15 min at room temperature in the absence or in the presence of 1% Triton X-100. Samples were supplemented with PMSF to a final concentration of 5 mM, subjected to SDS–PAGE, and visualized by autoradiography.

Preparation of antibodies

To generate specific antibodies against the ASF virus protein encoded by EP153R gene, a 15-amino-acid-long peptide (MYFKKKYIGLIDKNC), covering the sequence 1–15 corresponding to the N-terminal region of the protein, was synthesized. The peptide was conjugated to keyhole limpet hemocyanin (Pierce) via C-terminal Cys, with the linking agent sulfo-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Brinkley, 1992) and used to immunize rabbits. The immune serum obtained recognized pEP153R on Western blots.

Western blot

Protein samples were dissociated in Laemmli (1970) buffer, electrophoresed in a 7 to 20% polyacrylamide gel (SDS–PAGE), and transferred to nitrocellulose paper. After being blocked with 1% skim milk in TBS, the membranes were incubated overnight at 4°C with either anti-pEP153R or anti-pEP402R serum, diluted 1/100 or 1/5000, respectively, in TBS containing 0.1% Tween 20. Bound antibodies were detected with a second peroxidase-conjugated antiserum and luminol by the ECL detection system (Amersham) according to the manufacturer's instructions.

Hemadsorption assay

Cultures of swine alveolar macrophages or Vero cells were infected with either BA71V or vΔEP153R at a m.o.i. of 1 PFU per cell. At 12 hpi swine erythrocytes were added to a concentration of 2.5×10^6 cells/ml, and the manifestation of hemadsorption was monitored from 24 to 48 hpi by phase-contrast microscopy.

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